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(54) Title: GENE CONFERRING RESISTANCE TO THE ANTIBACTERIAL 4,5-DIHYDROXY-2-CYCLOPENTEN-1-ONE (DHCP), THE PROTEIN ENCODED BY SAME, AND APPLICATIONS THEREOF

(57) Abstract: The invention relates to a gene, *dep*, which confers resistance to the antibacterial activity of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP). The invention further relates to the putative protein encoded by *dep*, which is a hydrophobic, transmembrane efflux protein specific for DHCP. The inventin further relates to plasmids containing the *dep* gene, and to bacterial cells expressing *dep*. Furthermore, the invention provides applications for use in conferring resistance to antibacterial activity in organisms. The *dep* gene can be used to identify compounds which inhibit the efflux activity responsible for the resistance to DHCP or to compounds which are functionally equivalent to DHCP.

Gene conferring resistance to the antibacterial 4,5-dihydroxy-2-cyclopenten-1-one (DHCP), the protein encoded by same, and applications thereof

This patent application claims the benefit of U.S. Provisional Application No. 60/228,727, filed 8/29/2000. This earlier provisional application is hereby incorporated by reference.

In spite of a number of antibiotics available against a variety of bacteria, due to emergence of multiple drug resistant strains, the search for newer and more effective antibacterial compounds has continued. 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) (see Fig.1) is a compound having antibacterial activity against a variety of gram-positive and -negative bacteria, such as *Escherichia coli, Bacillus, Salmonella, Staphylococcus* etc. The process for manufacture and the properties of DHCP have been patented (Koyama *et al.*, 1999). It is prepared by the heat-treatment of uronic acid or its derivatives, wherein uronic acid is galacturonic acid, glucuronic acid, mannuronic acid or iduronic acid. It is also produced from roasted or parched vegetables, fruits, cereals, mushrooms, sea algae, cortex or cartilage. It has been demonstrated that this compound induces cancer cell differentiation and apoptosis. It has potential application as therapeutic or preventive agent against cancer and also as an antibacterial agent in antiseptics, dentrifices, cosmetics and bathing agents (Koyama *et al.*, 1999).

We have isolated a multicopy suppressor from an *E. coli* genomic library for the DHCP toxicity. The putative protein encoded by this gene showed high homology to known efflux proteins conferring resistance to a number of antibiotics including chloramphenicol, bicyclomycin and tetracycline. The gene was mapped at 37.5 min on the *E. coli* chromosome. It is designated as *dep* for DHCP efflux protein. However, the Dep protein does not confer cross-resistance to any of the antibiotics tested.

Prior Art References

United States Patent No. 6,087,401 to Koyama, et al. Cyclopentones, process for preparing same, and the use thereof.

This patent discloses a method of manufacturing 4,5-dihydroxy-2-cyclopenten-1-one (DHCP). It also describes the antibacterial activity of DHCP.

In contrast, the invention disclosed in the present provisional application relates to a gene, *dep*, that, when present in multiple copies in bacterial cells, confers resistance to the antibacterial activity of DHCP, thus rendering the bacteria resistant to killing by DHCP. The present application also describes the protein encoded by the *dep* gene.

European Patent EP 0 941 981 A1 to Koyama, et al. Cyclopentones, process for preparing same, and the use thereof.

This patent application relates to essentially the same subject matter as that described in United States Patent No. 6,087,401 to Koyama, et al.

United States Patent No. 6,111,145 to Kobayashi et al. Cyclopentenone derivative.

This patent relates to functionally equivalent ether derivatives of DHCP and discloses the biological activity of these derivatives.

European Patent Publication EP 1 000 923 A1 to Kobayashi et al. Cyclopentenone derivatives.

This patent application relates to essentially the same subject matter as that described in United States Patent No. 6,111,145 to Kobayashi et al.

United States Patent No. 6,136,854 to Koyama et al. Cyclopentenone derivative.

This patent relates to functionally equivalent ester derivatives of DHCP and discloses the biological activity of these derivatives.

European Patent Publication EP 0 976 717 A1 to Koyama et al. Cyclopentenone derivatives.

This patent application relates to essentially the same subject matter as that described in United States Patent No. 6,136,854 to Koyama et al.

Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. C. Wuchter, et al. Haematologica 85(7):711-21 (2000).

In acute leukemia, a multidrug-resistance (MDR) phenotype mediated by P-glycoprotein (P-gp) contributes to chemotherapy failure. This study investigated whether P-gp expression levels or functional P-gp activity was a better predictor of response to induction chemotherapy, relapse rate and overall survival in acute leukemia. The data demonstrated that the functional rhodamine-123- (rh123)-efflux assay was preferred over P-gp expression analysis by monoclonal antibodies in acute leukemia.

Increased drug delivery to the brain by P-glycoprotein inhibition. A.J. Sadeque, et al. Clinical Pharmacology & Therapeutics 68(3):231-7 (2000).

In vitro studies had demonstrated that the antidiarrheal drug loperamide is a substrate for the efflux membrane transporter P-glycoprotein. Although loperamide is a potent opiate drug, it does not opioid central nervous system effects, such as respiratory depression, when given to patients at usual doses. This study tested the hypothesis that inhibition of P-glycoprotein with quinidine would increase the entry of loperamide into the central nervous system, thus causing respiratory depression. The results demonstrated that although loperamide produced no respiratory depression when used alone, respiratory depression was seen when loperamide was administered with quinidine.

Expression of the multidrug-resistance-associated protein in myelodysplastic syndromes. S. Poulain, et al. British Journal of Haematology 110(3):591-8 (2000).

In myelodysplastic syndromes (MDS), P-glycoprotein (P-gp) expression is associated with drug resistance, while the clinical significance of the multidrug resistance-associated protein (MRP1) is unclear. In this study of bone marrow from patients with MDS, expression of MRP1 was correlated with disease stage in MDS. With respect to P-gp, discordant expression/function of MRP1 was found in some cases, suggesting the existence of nonfunctional transport proteins in MDS. MRP1 expression did not appear to be a prognostic factor in MDS.

Soft tissue leiomyosarcomas and malignant gastrointestinal stromal tumors: differences in clinical outcome and expression of multidrug resistance proteins. B. E. Plaat, et al. Journal of Clinical Oncology 18(18):3211-20 (2000).

In this study, parameters associated with multidrug resistance (MDR) were compared between soft tissue leiomyosarcomas (LMS) and malignant gastrointestinal stromal tumors (GIST). Immunohistochemistry was used to detect P-glycoprotein (P-gp), multidrug resistance protein (MRP(1)), lung resistance protein (LRP), and c-kit. The results demonstrate that LMS

patients have better survival rates compared to GIST patients, and the pattern of metastasis differs between the two patient groups. The expression of the MDR proteins tested is less pronounced in LMS than in GIST.

Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. M.G. Surette, et al. Proc. Natl. Acad. Sci. 96:1639-44 (1999).

In bacteria, the regulation of gene expression in response to changes in cell density, called quorum sensing, is dependent on hormone-like molecules known as autoinducers that are produced by the bacteria and accumulate in the external environment as the bacterial cell population increases. The marine bacterium *Vibrio harveyi* has been shown to have two parallel quorum sensing systems, each composed of a sensor-autoinducer pair. The two different autoinducers belonging to each system have been termed autoinducer 1 (AI-1) and autoinducer 2 (AI-2). The identification and analysis of the genes responsible for AI-2 production in *E. coli*, *S. typhimurium*, and *V. harveyi* is reported.

Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. A. L. Schaefer, et al. Journal of Bacteriology 178:2897-2901 (1996).

In *Vibrio fischeri*, luminescence genes are activated by the transcription factor LuxR in combination with a diffusible signal compound known as the autoinducer. This study analyzed the ability of a number of autoinducer analogs to interact with LuxR.

Regulation of quorum sensing in *Vibrio harveyi* by LuxO and Sigma-54. B.N. Lilley and B. L. Bassler. Molecular Microbiology 36(4):940-954 (2000).

The bioluminescent marine bacterium *Vibrio harveyi* controls light production (*lux*) by a quorum-sensing circuit. This study demonstrates that the response regulator protein LuxO functions as an activator protein via interaction with the alternative sigma factor, σ^{54}. Since LuxO is responsible for repression of the luciferase structural operon (*lux*CDABEGH), these results suggest that LuxO, together with σ^{54}, functions to activate a negative regulator of luminescence.

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All references cited herein are incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is the chemical structure of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP).

FIG. 2A is a graphical representation of the effect of DHCP concentration on the growth of *E. coli*.

FIG. 2B is a graphical representation of the effect of DHCP concentration on the survival of *E. coli*.

FIG. 3 is a restriction mapping of the plasmid pSP001 showing the DNA fragments conferring resistance to DHCP.

FIG. 4 is a comparison of the amino acid sequence of the polypeptide encoded by *dep* with the proteins encoded by *cmr*, *cmrA*, *cmx*, *cmlv*, *bcr*, *bmr3*, *yjcC*, and *tet*.

FIG. 5 is a comparison of the hydropathic profiles of the putative proteins encoded by *dep*, *cmr*, and *cml*.

FIG. 6 is a nucleotide sequence showing the DNA sequence of a region of the *E. coli* genome containing the sequence of the *dep* gene.

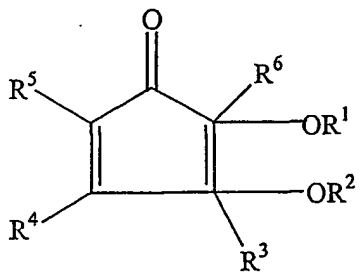
FIG. 7 is a nucleotide sequence showing the isolated DNA sequence of the *dep* gene.

BRIEF DESCRIPTION OF THE INVENTION

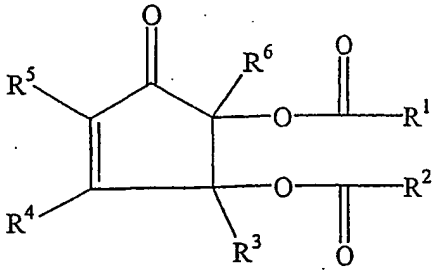
The provisional application describes the cloning of a gene encoding a transmembrane protein from *E. coli*. This protein, when expressed from a multi-copy plasmid, functions to transport 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) out of the cell.

DHCP and functionally equivalent compounds are represented by the formulas [I] and [II] and include optically active compounds thereof. In Formula I, R^1 and R^2 are the same or different and each of them is hydrogen, a straight or branched alkyl group, a straight or branched alkenyl group, an aromatic group, an aromatic-aliphatic group, with the proviso that R^1 = a benzyl group and R^2 = H is excluded.

See References 3 and 4. R^3-R^6 are independently hydrogen or an alkyl group, preferably a lower alkyl group such as a C_1-C_6 alkyl.



Formula I



Formula II

In Formula II, R^1 and R^2 are the same or different and each of them is hydrogen, a straight or branched alkyl group, a straight or branched alkenyl group, an aromatic group, an aromatic-aliphatic group, with the proviso that the case where $R^1 = R^2 = CH_3$ is excluded. See References 5 and 6. R^3-R^6 are independently hydrogen or an alkyl group, preferably a lower alkyl group such as a C_1-C_6 alkyl.

DHCP is shown to possess anti-bacterial activity; it inhibits cell growth at a concentration of 350μM or higher. At lower concentrations, it causes cells to elongate and grow poorly. To determine if *E. coli* is naturally resistant to DHCP, a library of *E. coli* genomic DNA fragments was transformed into strain JM83 and grown on agar plates containing 400μM DHCP. Colonies that were capable of growing on this medium were isolated. DNA was isolated from these colonies to identify and sequence the cloned genomic fragment that specified resistance. Four genes were found in the fragment that conferred resistance. Inactivation of various combinations of these four genes led to the conclusion that ORF389 was responsible for conferring resistance. This was confirmed by cloning ORF389 by itself into pUC19 (a multi-copy plasmid) and transforming strain JM83. The resultant cells were resistant to DHCP.

Comparison of the nucleotide sequence of ORF389 with the *E. coli* gene database showed that it was similar to known efflux proteins involved in conferring resistance to chloramphenicol and other antibiotics. Further analysis of the predicted structure of the protein encoded by ORF389 suggested that it was a membrane protein; it possesses multiple transmembrane domains and shares structural similarity with the aforementioned chloramphenicol efflux polypeptides.

To determine if ORF389 was capable of conferring resistance to other antibiotics such as chloramphenicol, spectinomycin, and tetracycline, the transformed JM83 cells containing the pUC19/ORF389 plasmid were plated on media containing these antibiotics. The presence of ORF389 failed to confer resistance to any antibiotic other than DHCP, suggesting that the efflux activity of the Dep protein is specific for DHCP.

It is important to note that ORF389 confers resistance to DHCP only when it is present in multiple copies in the cell. The gene is naturally found in the genome of *E. coli* cells, but it is present in single copy. Such cells are susceptible to the antimicrobial activity of DHCP. When ORF389 is cloned into pUC19 and introduced into JM83 cells, it is present in multiple copies (up to several hundred copies of the gene per cell), since pUC19 is maintained in up to several hundred copies per cell. Only when the gene dosage is increased, is resistance to DHCP found. The mechanism of resistance is simply increased efflux activity arising from the increased expression of the efflux protein in the transformed cells.

It should be noted that due to the degeneneracy of the genetic code, the nucleotide sequence encoding an efflux protein that is responsible for conferring resistance to DHCP or a compound functionally equivalent to DHCP may vary from the nucleic acid sequence disclosed herein.

ADDITIONAL DESCRIPTION OF THE INVENTION

Mode of Action

DHCP is a compound that exhibits antimicrobial and anti-tumor activity. It is made by heating various uronic acids (e.g., glucuronic acid, galacturonic acid, mannuronic acid). The inventors have cloned a gene from *E. coli* that encodes a protein which is capable of transporting DHCP out of the cell. This transport protein shows sequence similarity with known efflux proteins that function to transport antibiotics such as chloramphenicol out of the cell. It has been shown that organisms which overexpress the transport protein become resistant to DHCP, probably because they are able to efficiently transport DHCP. Overexpression of the transport protein arises from the presence of multiple copies of the gene, rather than increased expression from the endogenous gene in *E. coli*. In other words, all *E. coli* possess a single copy of the transport gene. However, the level of transport protein expression from a single copy of the gene is insufficient to confer resistance to DHCP. The inventors have cloned the gene into a high copy number plasmid, pUC19, which is maintained in *E. coli* cells at 200-500 copies per cell. Thus, transformed *E. coli* containing this plasmid construct will possess 200-500 copies of the transport gene, and protein expression from multiple copies is greater than from a single copy. These transformed cells are resistant to DHCP.

The general mode of action of DHCP requires that it enter the target cell. Resistance to DHCP can occur if DHCP is transported out of the cell as fast as or faster than it enters the cell. Given that, the concentration of DHCP within the cell can never accumulate to a toxic dose and the cell is resistant to the antimicrobial effects of the compound. Apparently, the transport protein encoded by gene disclosed does not transport DHCP very efficiently, or the amount of transport protein expressed from the endogenous gene is very low. In either case, the presence of more transport protein (arising from many copies of the gene) will result in more efficient transfer of DHCP out of the cell.

Applications

An important application of the gene of the invention will be its use in studies to identify inhibitors of efflux activity. Such inhibitory compounds will function to block the transport activity. Thus a microbe or a tumor cell that is resistant to DHCP can be made to be more sensitive to the compound by preventing the resistant cell from transporting the compound back out. It is also conceivable that inhibitors of the transport gene of the invention may also be active in blocking transport of other efflux proteins such as the efflux proteins that transport chloramphenicol, or the P glycoprotein family of multiple drug resistant proteins. The P glycoproteins are expressed in many tumor cells, making these tumors resistant to chemotherapy agents. Abstracts regarding studies of P glycoproteins are referenced above.

DETAILED DESCRIPTION OF THE DRAWINGS

FIG. 1 is the chemical structure of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP).

FIG. 2 Effect of DHCP concentrations on the growth of *E. coli.*

A. The JM83 cells were grown in LB medium up to Klett unit of 50 and DHCP was added at various concentrations (0-400 μM). After the growth reached to Klett unit of 90-100, cells were diluted with medium containing respective concentrations of DHCP and growth was further monitored. DHCP concentration: 0 μM, open squares; 50 μM, closed diamonds; 100 μM, open circles; 250 μM, open diamonds; 400 μM closed squares.

B. Overnight grown cells of *E. coli* JM83 were diluted appropriately and plated on LB plates containing different concentrations of DHCP (0-350 μM). The number of colonies on the plate without DHCP was taken as 100% and the other numbers were expressed as relative percentages.

FIG. 3 Restriction mapping of the plasmid pSP001 conferring resistance to DHCP. Four ORFs comprising the DNA fragment (5.2 kb) conferring resistance to DHCP and the flanking ORFs are shown. The orientation of each ORF is marked with an arrow. The restriction enzyme sites are also shown. The ORFs are not drawn to scale. The plasmid pSP001 containing the DNA fragment conferring resistance to DHCP was digested with restriction enzymes to disrupt each of four ORFs, religated and transformed into JM83 cells. The transformants were then examined for their sensitivity to DHCP (400 μM). The enzymes used for digestion were: for *purR*: *Mlu*I for *ydhB; Nru*I-*Eco47*III, for ORF389, *purR* , and *ydhB: Nru*I and *Sma*I, for ORF389: *Ava*I and for *purR* and *ydhB*: *Mlu*I and *Nru*I. For construction of plasmid with ORF389 (*dep*), the plasmid pSP001 was digested with *Sma*I and *Msc*I, the fragment was purified and cloned into pUC19 to yield plasmid pSP007.

FIG. 4 The sequence homology between Dep, Cmr, CmrA, Cmx, CmlV, BcR, Bmr3, YjcC and Tet. Identical and similar sequences are marked with black and gray boxes, respectively. The consensus sequences for transmembrane proteins are marked with dotted lines and are represented as I, II, and III stretches.

FIG. 5 Hydropathic profiles of Dep (A), Cmr from *Rhodococcus faciens* (B) (6) and Cml from *Streptomyces lividans* (C) (8). Horizontal bars indicate predicted transmembrane regions.

FIG. 6 is a nucleotide sequence showing the DNA sequence of a region of the *E. coli* genome containing the sequence of the *dep* gene. This region of the *E. coli* genome is available at Accession No. AE000261 U00096. The sequence shown is that of nucleotides 4381-8280. The *dep* gene is encoded by nucleotides 4627-5838. The *dep* sequence is shown in brackets.

FIG. 7 is a nucleotide sequence showing the isolated DNA sequence of the *dep* gene. The plasmid pSP007 was confirmed to contain the *dep* gene by obtaining DNA sequence data from one end of the 1.7 kb insert. Sequence data obtained in this manner matched the first.

Detailed Description of Experimental Work

Effect of DHCP on the growth of *E. coli*

The *E. coli* wild-type strain JM83 [F'*ara*Δ (*lac-proAB*) *rpsL*(*str*^{r})](Yanisch-Perron *et al.*, 1985) was grown in Luria broth (LB). Media were supplemented with ampicillin (final concentration of 50 μg/ml) whenever required. To check the effect of DHCP on the growth of *E. coli*, cells grown overnight in LB medium were diluted into fresh LB medium. After the growth reached to the Klett unit of 50, DHCP was added at various concentrations (0-400 μM) and growth was further monitored. After it reached to the Klett unit of 90-100, it was diluted 10-fold into media containing respective concentrations of DHCP. Fig. 2A shows the effect of different concentrations of DHCP on *E. coli*. The growth was slowed after 3 h of incubation in the presence of 50 μM DHCP, but it reached the maximum density after 8 h, similar to that without DHCP. The cells grew more slowly after 3 h incubation with 100 μM of DHCP and the maximum cell density was lower than that without DHCP. In the presence of 250 μM DHCP, growth was severely impaired after 3 h of incubation and cells stopped growing after 5 h. In the presence of 400 μM DHCP, cell growth stopped after 4 h of incubation. Microscopic examination of the cells grown with 250 μM DHCP for 8 h showed that the cells were elongated

forming filaments, which are approximately 15-fold longer than the control cells. DAPI (diamidino phenylindole)(Hiraga *et al.*, 1989) staining of these cells showed that the chromosomal condensation of the cells might be impaired by DHCP (data not shown).

To check the colony formation ability of *E. coli* at various concentrations of DHCP, cells grown overnight in LB medium were diluted appropriately and plated on LB plates containing DHCP (0-350 μM). After incubation at 37 ^{0}C, the number of colonies on each plate were counted. The number of colonies on the control plate without DHCP was taken as 100% and the other numbers were expressed as relative percentages (Fig. 2B). In the presence of 300 μM DHCP, 100-fold decrease in the colony numbers was observed. When 1x10^4 cells were plated on LB medium containing 350 μM DHCP, no colonies were obtained.

Screening of an *E. coli* genomic library for genes conferring resistance to DHCP

In order to examine if *E. coli* contains a gene(s) that confers resistance to DHCP, the *E. coli* genomic library was screened. The construction of *E. coli* genomic library was described previously (Lu and Inouye, 1998). The partially digested *Sau*3AI chromosomal DNA fragments from *E. coli* JM83 were cloned into the *Bam*HI site of pUC19. The JM83 cells were transformed with the genomic library. Transformants were isolated for their ability to grow on DHCP (400 μM) containing LB plates at 37 ^{0}C. Plasmid DNA was isolated from the resistant colonies, purified and retransformed into JM83 cells to confirm its ability to confer resistance to DHCP. The plasmid was designated as pSP001 and was found to contain a 5.2-kb DNA fragment. This fragment was sequenced from both ends using Sequenase and BLAST search was carried out for the analysis of homology of this fragment with the entire *E. coli* genome. It was found that this DNA fragment is located at 37.5 min on the *E. coli* chromosome and contains four ORFs (Fig. 3): ORF389, *purR* encoding purine synthesis repressor, *ydhB* encoding a homologue of the *cyn* operon transcriptional activator and *ydhC* encoding a homologue of bicyclomycin resistance protein (Berlyn *et al.*, 1996).

To determine which gene is responsible for conferring resistance to DHCP, several deletion constructs were prepared as shown in Fig. 3. Disruption of *purR*, *ydhB* and both *purR*

and *ydhB* had no effect on the resistance to DHCP (constructs pSP002, pSP003 and pSP006, respectively). However, disruption of ORF389 with *purR* and *ydhB* (pSP004) as well as disruption of ORF389 alone (pSP005) resulted in loss of DHCP resistance. We thus cloned ORF389 separately in pUC19 (pSP007), transformed the resultant plasmid in JM83 and checked sensitivity to DHCP. This plasmid conferred resistance to DHCP. These results clearly demonstrate that ORF389 is responsible for resistance to DHCP when cloned in a multicopy plasmid and further work was carried out using the plasmid pSP007. The ORF389 was named as *dep* – DHCP efflux protein (see below).

Homology analysis of ORF389 with other genes conferring drug resistance

Using BLAST-homology search computer program, we carried out a homology search for the putative protein encoded by *dep*. Fig. 4 shows nine proteins showing significantly high homology with Dep. Half of these proteins confer resistance to chloramphenicol. The proteins showing the highest degree of homology include: Cmr from *Rhodococcus fasciens* (Desomer *et al.*, 1992), CmrA from *R. erythropolis* (Nagy *et al.*, 1997), Cml from *Streptomyces lividans* 1326 (Dittrich *et al.*, 1991), Cmx from *Corynebacterium striatum* (Accession no. U72639), and CmlV from *S. venezuelae* ISP5230 (Mosher *et al.*, 1995). As seen from Fig. 4, Dep has the highest degree of homology with Cmr, product of chloramphenicol resistant gene (*cmr*) as compared to other proteins. Cmr protein was shown to contain three consensus sequences defined by Rouch *et al.* (1990) for transmembrane proteins. These sequences are at similar positions with respect to the predicted transmembrane domains. These are marked in Fig. 6 with dotted lines and are designated as I, II, III. In case of Dep, the first stretch (I) comprising of LP is completely homologous with the stretch defined by these authors. The second stretch (II) shows 50% similarity with that of Cmr protein and the third stretch (III) is homologous between these two proteins except for one residue. According to the model proposed by Rouch *et al.* (1990), the stretches I and III are located on the outside of the cytoplasmic membrane and the stretch II is located on the inside of the membrane. The positions of the membrane loops for the putative

protein encoded by *qacA* were ascertained by inspecting the antigenic index profile and turn prediction. Such regions have a high antigenic index and turn probability (Rouch *et al.*, 1990).

In addition to homology in the primary sequences, the hydropathic profile of Dep (Fig. 5A) is significantly similar to those of Cmr of *R. faciens* (Desomer *et al.*, 1992) (Fig. 5B) and Cml of S. *lividans* (Dittrich *et al.*, 1991) (Fig. 5C). Dep is predominantly hydrophobic and probably contains 12 predicted transmembrane α-helices (Fig. 5A).

The other proteins homologous to Dep include BcR (bicyclomycin- resistance protein) from *E. coli* (Bentley *et al.*, 1993), Bmr3 from *B. subtilis* involved in the multiple drug efflux pump conferring resistance to puromycin, tosufloxacin, norfloxacin (Ohki and Murata, 1997), Tet from *Staphylococcus hyicus* conferring tetracycline resistance (Schwarz *et al.*, 1992) and YjcC conferring tetracenomycin-resistance (Accession no. D90826) (Fig. 4). All of these are efflux proteins, which is one of the most common mechanisms for drug resistance. We speculate that *dep* encodes a putative efflux protein that forms a cytoplasmic channel specific for DHCP. The homologies are more prominent towards the N-terminal end of the proteins, which also is a common feature for efflux proteins (Desomer *et al.*, 1992).

Measurement of minimum inhibitory concentrations for cells harboring pUC19 and pSP007

Since Dep shows homology to efflux proteins for multiple drug resistance, we checked if it confers resistance to other antibiotics as well. The *E. coli* wild-type cells harboring pUC19 or pSP007 plasmid were grown overnight in LB medium containing ampicillin. The cells were diluted 10- and 1000- times, and 5 μl of each dilution (corresponding to $3.5x10^5$ cells and $3.5x10^3$ cells, respectively) was spotted on LB plates containing serial dilutions of kanamycin, chloramphenicol, spectinomycin, tetracycline and DHCP. Plates were incubated at 37 ^{0}C for 20 h. As seen from Table1, pSP007 did not confer significant cross-resistance to any of the antibiotics tested. The MIC values for cells harboring pUC19 and pSP007 were same for spectinomycin, chloramphenicol and tetracycline. The MIC value was two times higher for kanamycin for the cells harboring pSP007 than the cells with pUC19. The MIC value for DHCP

on the other hand was 8 times higher for the cells harboring pSP007 than that for the cells with pUC19. It is interesting that Dep did not confer resistance to chloramphenicol, in spite of the high homology to *cmr*.

Table 1. Minimum inhibitory concentrations (MICs) of various antibiotics for *E. coli* JM83 cells harboring pUC19 and pSP007.

	MICs (μg/ml)				
	kanamycin	spectinomycin	chloramphenicol	tetracycline	DHCP
cells with pUC19	25	12.5	6.25	3.125	25
cells with pSP007	50	12.5	6.25	3.125	200

MICs for both dilutions of the cells ($3.5x10^5$ and $3.5x10^3$ cells) were the same.

In the Claims

We claim:

1. A DHCP efflux protein, which is specific for 4,5-dihydroxy-2-cyclopenten-1-one (DHCP).

2. The protein of Claim 1, which is a transmembrane protein that forms a cytoplasmic channel specific for efflux transport of DHCP.

3. The protein of Claim 1, which confers resistance to DHCP.

4. The protein of Claim 1, which protein is from *E. coli*.

5. The protein of Claim 1, which protein does not confer cross-resistance to the any of the following antibiotics: chloramphenicol, spectinomycin and tetracycline.

6. The protein of Claim 1, which protein possesses 13 predicted transmembrane-spanning α-helices.

7. A gene encoding *dep*, the DHCP efflux protein.

8. The *dep* gene of Claim 7, wherein the *dep* gene is from *E. coli*.

9. The gene of Claim 7, wherein said gene confers resistance to DHCP or a functionally equivalent compound when present in multiple copies in a bacterial cell.

10. A plasmid comprising the *dep* gene, which plasmid confers expression of multiple copies of the *dep* gene in bacteria cells that have been transformed with said plasmid.

11. The plasmid of Claim 10, which plasmid confers resistance to DHCP and does not confer cross-resistance to any of the following antibiotics: chloramphenicol, spectinomycin and tetracycline.

12. Bacteria cells containing multiple copies of the plasmid of Claim 10.

13. The bacteria cells of Claim 12, which bacteria cells are resistant to DHCP.

14. A method which uses the gene of Claim 7 to identify a compound which inhibits efflux activity responsible for resistance to DHCP or a functionally equivalent compound.

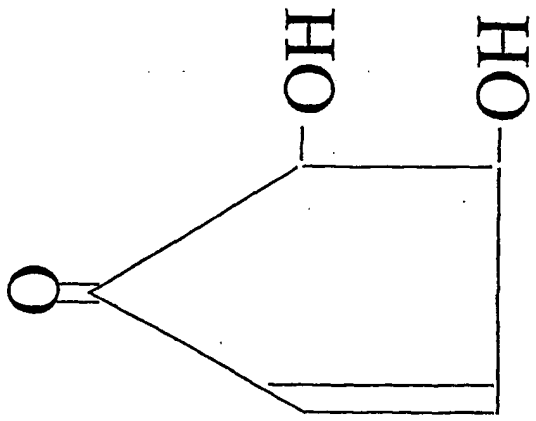


Fig. 1

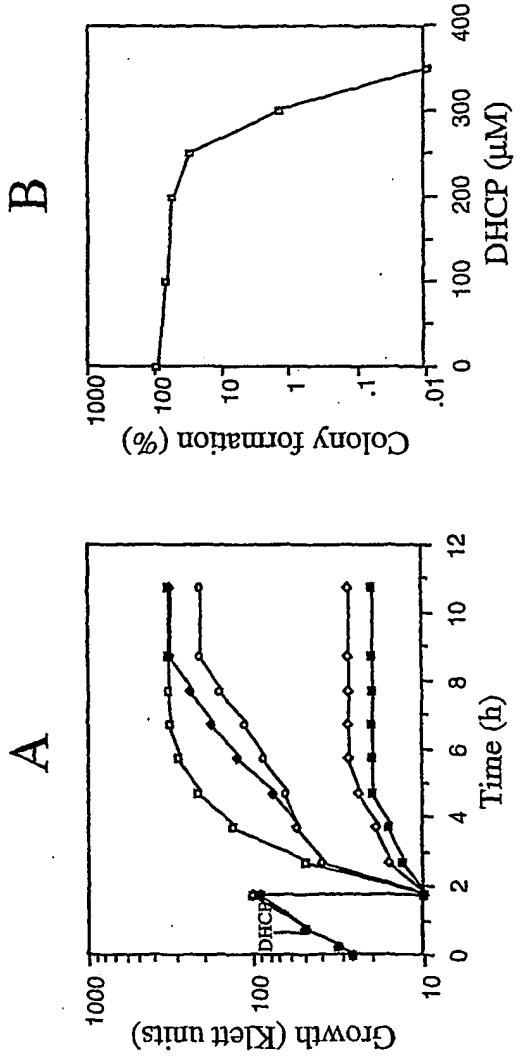

A
Growth (Klett units)
1000
100
10
DHCP
0
2
4
6
8
10
12
Time (h)
B
Colony formation (%)
1000
100
10
1
.1
.01
0
100
200
300
400
DHCP (μM)

Fig. 2

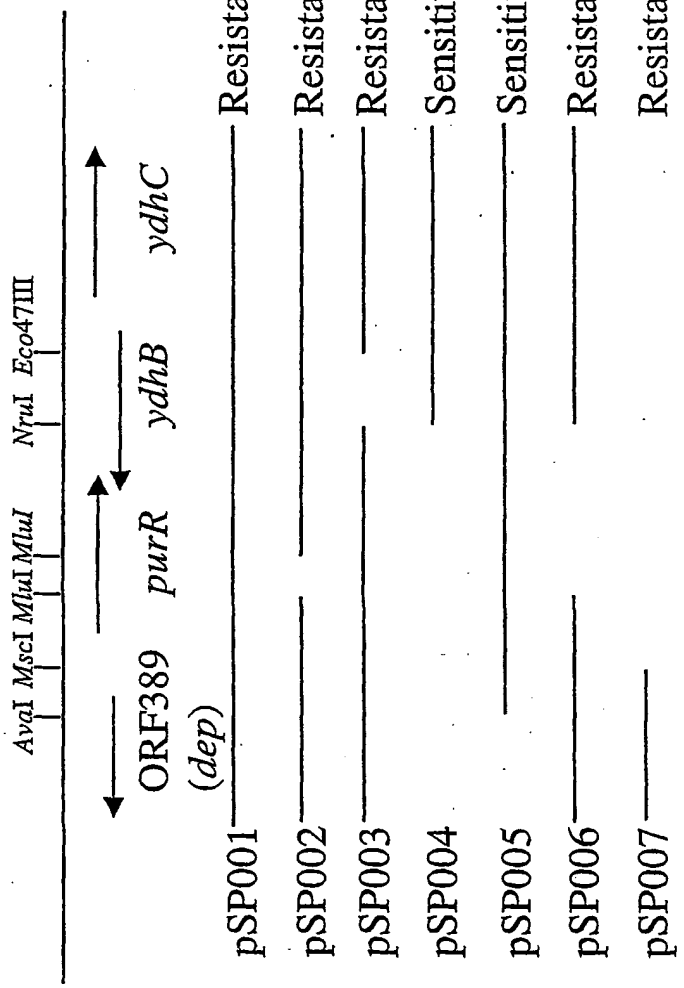

AvaI MscI MluI MluI NruI Eco47III
ORF389 (dep)
purR
ydhB
ydhC
pSP001 Resistant
pSP002 Resistant
pSP003 Resistant
pSP004 Sensitive
pSP005 Sensitive
pSP006 Resistant
pSP007 Resistant

Fig. 3

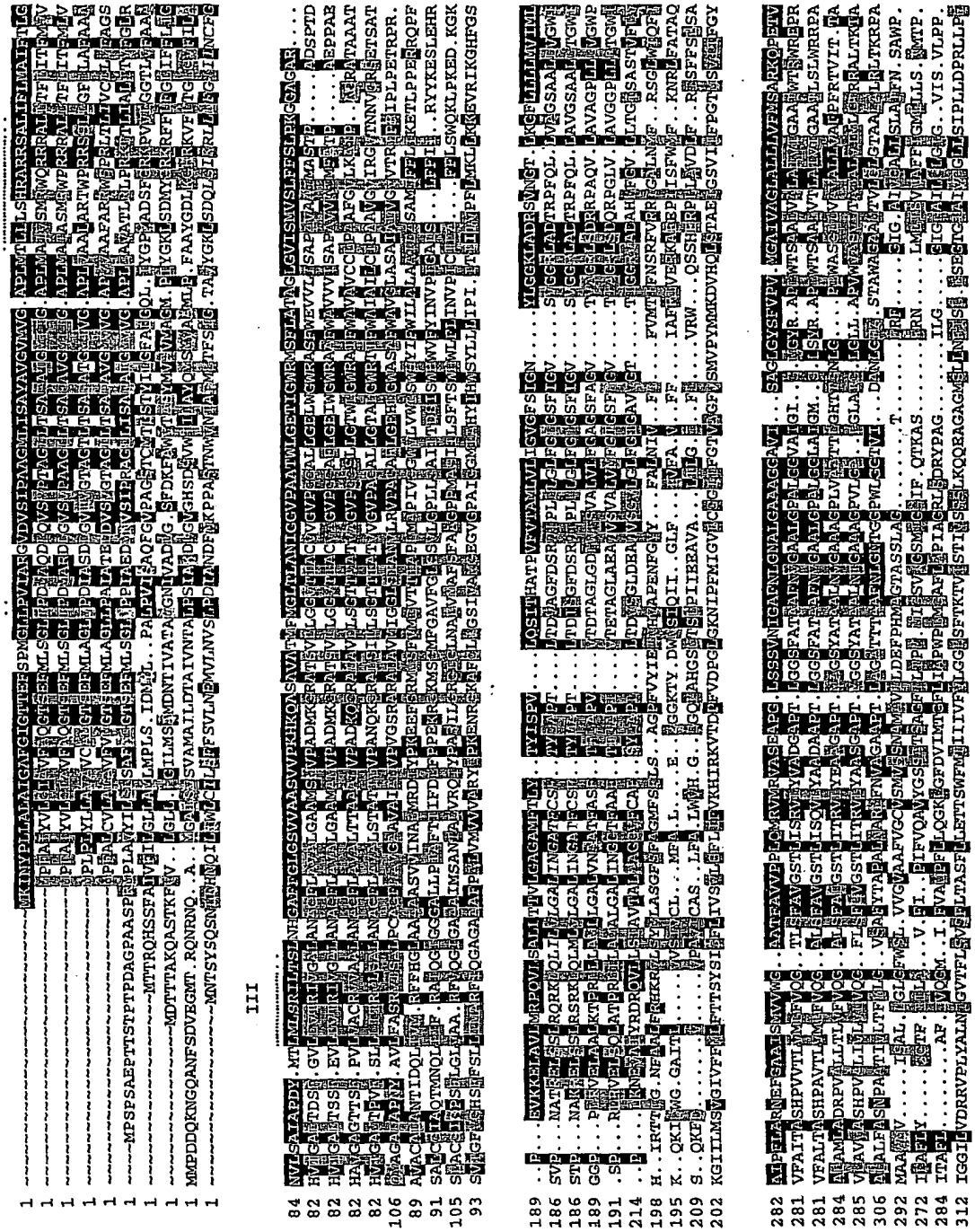


Fig. 4

Fig 4 (cont)

```
Dep     385 CVANS~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cmr     384 PALD[illegible]VPP~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CmrA    384 SVQE[illegible]VPA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cml     386 APADATR~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cmx     388 AEAN~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CmlV    409 PGHVVARSRGAGGTTPSEPARGKATSSC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
BcR     372 ......MIW...SIAFCATS..SILFCLYASRPKKR~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Bmr3    354 ...[illegible]ARVW..LTVFMM.ISGFGVGFNFSLL.P..AASMNDLEPRFR.GTANSTNSFLRSFGMTLGVTIFGTVQTNVFTNKLNDAFSGMK.G..SAGSGA.AQNIGDPQE
YjcC    366 ...H[illegible]KPI...IAVIMMALCG.G.GFGFFLS.PNQRALMSS.APTTRSGAASGVLGISRILGQTTGATL...VAFCLYLSSDHGAEIALRIGIFIAFAGLYGQFVAFAE.
Tet     421 EVD[illegible]STYLYSNLLLLFSGIIVISWLVTLNLYKHSQRDF~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Dep     390 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cmr     392 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CmrA    392 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cml     393 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cmx     392 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CmlV    437 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
BcR     397 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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Fig. 4 (cont.)

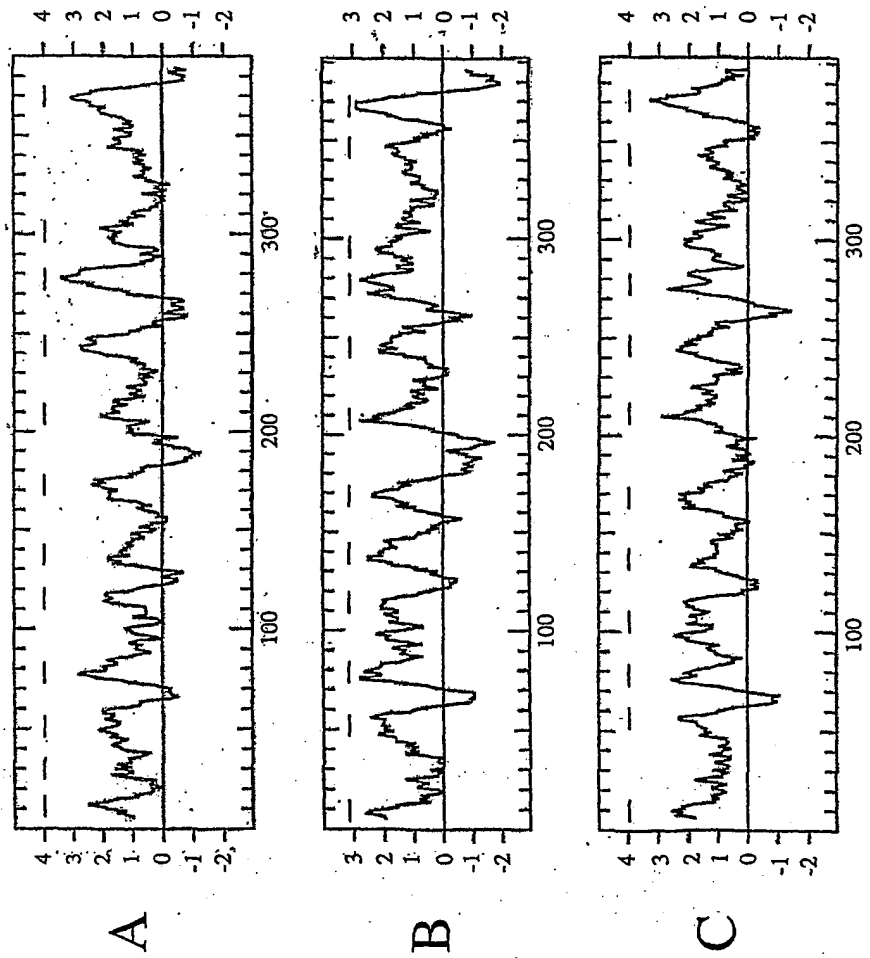

Fig. 5

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FIG.6

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FIG. 1

SEQUENCE LISTING

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PHADTARE, SANGITA
YAMANAKA, KUNITOSHI
KATO, IKUNOSHIN

<120> ANTIBACTERIAL ACTIVITY OF 4.5 DIHYDROXY-2-CYCLOPENTAN-1-ONE (DHCP) AND CLONING A GENE CONFERRING DHCP RESISTANCE IN ESCHERICHIA COLI

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INTERNATIONAL SEARCH REPORT

Internat' application No.
PCT/U 26961

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 31/12

US CL : 530/350; 536/23.1; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: MEDLINE, BIOSIS, WPIDS, JAPIO, USPAT, EMBASE, DGENE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Database HCAPLUS on STN (Piscataway, NJ, USA), No.135:119459, 'Antibacterial Activity of 4, 5-dihydroxy-2-cyclopenten-1-one (DHCP) and Cloning of a Gene Conferring DHCCP Resistance in Escherichia coli, Journal of Molecular Microbiology Biotechnology', abstract, Phadtare et al., 2001.	1-14
A	US 6,087,401 A (KOYAMA ET AL) 11 July 2000 (11/7/00), see entire document.	1-14
A	US 6,228,892 B1 (TOMINAGA ET AL) 08 May 2001 (08/05/01), see entire document.	1-14
A	WO 99/36383 A1 (TAKARA SHUZO CO., LTD), 22 JULY 1999 (22/07/99), see entire document.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

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(54) Title: GENE CONFERRING RESISTANCE TO THE ANTIBACTERIAL 4,5-DIHYDROXY-2-CYCLOPENTEN-1-ONE (DHCP), THE PROTEIN ENCODED BY SAME, AND APPLICATIONS THEREOF

(57) Abstract: The invention relates to a gene, *dep*, which confers resistance to the antibacterial activity of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP). The invention further relates to the putative protein encoded by *dep*, which is a hydrophobic, transmembrane efflux protein specific for DHCP. The inventin further relates to plasmids containing the *dep* gene, and to bacterial cells expressing *dep*. Furthermore, the invention provides applications for use in conferring resistance to antibacterial activity in organisms. The *dep* gene can be used to identify compounds which inhibit the efflux activity responsible for the resistance to DHCP or to compounds which are functionally equivalent to DHCP.